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In order to develop an extremely sensitive test for chemicals that induce chromosomal rearrangements, a polymerase chain reaction (PCR) assay has been optimized for the single-molecule detection of a translocation-containing human DNA sequence. We have used this assay for rearrangements between the *bcl-2* proto-oncogene and the J_H immunoglobulin gene region to test for this lymphoma-associated translocation in genotoxin-treated cultured cells and in peripheral blood mononuclear cells. Our most important findings from this study are that approximately half of healthy adults contain this translocation in their blood at levels detectable with our ultrasensitive assay; that there is an age-related increase in the frequency of this translocation; and that some individuals have much higher levels of this translocation than their age-mates. This may reflect environmentally hazardous exposures or genetic defects and an increased risk of developing cancer.

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Melanie Ehrlich 9/4/96

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5. INTRODUCTION

Tests for mutagenicity have been used to screen various chemicals for their ability to cause point mutations and this is often done to try to predict their potential carcinogenicity (1,2). However, such a use of tests for mutagenicity can overestimate as well as underestimate carcinogenic potential and should be supplemented with other types of assays. We have been establishing tests to detect a type of change in DNA, other than point mutations, that has more recently been shown to frequently be one of the steps in carcinogenesis, namely, chromosomal rearrangements (3). We have used recent molecular biology techniques to develop new methods to screen chemical mixtures for their ability to induce rearrangements of chromosomes. We also compared those methods to classical cytogenetic techniques using cultured human cell populations as the test system.

The prominent role of chromosomal rearrangements, especially translocations and deletions in cancer formation has only lately been widely appreciated (3-5). Chemical as well as "spontaneous" carcinogenesis and generation of birth defects often involve various types of rearrangements of human chromosomes (3-5). These are also a major source of spontaneous abortions. For example, translocations and deletions too small to be visualized by microscopic techniques are important contributors to genetic dysfunction but could not be analyzed until the advent of modern molecular biology methods.

Paternal, as well as maternal, chromosome damage has been implicated in environmentally caused birth defects and infertility (6). Among the agents that have been suspected to cause such damage are Agent Orange, the herbicide 2,4,5-T, and other phenoxy herbicides. Evidence for this comes from laboratory and epidemiological data (7,8). Furthermore, chromosomal rearrangements, birth defects, and cancer are linked together in several groups of genetic diseases in which a high rate of chromosomal rearrangements is coupled with very high rates of cancer as well as disorders of the skin, nervous system, and immune mechanisms (9). This observation is consistent with the major contribution of chromosomal rearrangements to both congenital defects and cancer.

The ability of various chemicals to induce chromosomal rearrangements has been studied less than their mutagenicity because sensitive assays for these rearrangements are new and the causative role of chromosomal rearrangements in carcinogenesis was firmly established only recently (3-5). Most mutations do not result in changes in protein structure due to the degeneracy of the triplet code, the ability of many amino acids to substitute for one another at a given position in a protein, and the occurrence of many mutations in unimportant regions of chromosomes. In contrast, a much higher percentage of chromosomal rearrangements will be carcinogenic or, if occurring in the male or female germline, will cause birth defects because rearrangements have a high probability of disrupting genes or gene regulatory regions and such disruptions almost always lead to abnormal protein synthesis. Therefore, tests for chromosomal rearrangements could, in many cases, be a better predictor of chemically-caused genetic damage to humans than is general mutagenicity testing.

To test for the ability of suspect chemicals to cause a chromosome translocation that is known to be one of the causes of human cancer, one of the tests that we have used is the polymerase chain reaction (PCR). This is an *in vitro* reaction conducted to replicate, up to a million-fold or more, a short DNA region in the presence of a vast excess of other DNA sequences. It involves the use of the DNA sample as a template and two primers that face each other, one each to replicate the top and bottom strands of the DNA sequence of interest in the presence of a vast excess of other DNA sequences. PCR utilizes multiple cycles (usually about 20 - 30) to amplify the DNA. Each cycle involves the following steps: DNA denaturation by heating, annealing of a pair of primers to the DNA template, and replication catalyzed by a thermostable DNA polymerase. The type of PCR assay we have developed is one in which two rounds of PCR are conducted which increases the sensitivity and specificity of the reaction and eliminates interference from the false positive reactions that so often interfere with this technique. When two rounds of PCR are performed, one pair of primers is used for 20 - 45 cycles to obtain a DNA product and then more primer and DNA polymerase are added for 20 - 45 more cycles. With the technique used here, the two rounds are

conducted with different pairs of primers. The primers used for the second round are internal to the pair used for the first round. With this kind of PCR (nested PCR) one DNA fragment is amplified in the first round. Then, a subsequence of that DNA fragment is amplified in the second round. Obtaining the expected sizes of DNA fragments from *both* rounds is an excellent way to verify that the PCR is amplifying the intended sequence and not some nonspecific sequence.

As little as a single DNA molecule can be detected by PCR followed by blot hybridization (10). Generally when such exquisite sensitivity is achieved, the amount of total DNA present, including nonspecific DNA sequences, is not very high. A great excess of such nonspecific DNA can interfere with the amplification of the targeted DNA sequence and the detection of the specific amplification product. If a very large amount of extraneous DNA is present during such single-molecule detection by PCR, blot hybridization is usually employed to visualize only the desired PCR product (11). We wanted to obtain this same level of sensitivity in the presence of 10^6 genome equivalents of human DNA not containing the exponentially amplifiable sequence of interest using a procedure more amenable to routine analysis than one involving blot hybridization.

In our project we used such a method that employed 22 cycles of amplification with one set of primers for the target sequence (first round of PCR) followed by another 20 cycles (second round of PCR) in which one of the original primers is replaced by a radiolabeled primer complementary to part of the amplified sequence (semi-nested PCR). With this procedure, which should be of use in many types of amplification reactions, we were able to quantitate the number of target molecules in the range of 1 to 15 amplifiable molecules in the original sample.

The first targeted DNA in this study was the junction fragment containing the translocated *bcl-2* proto-oncogene region from human chromosome 18 and J_H DNA sequences from the immunoglobulin DNA portion of chromosome 14. This translocation, t(14;18), is observed in a majority of follicular lymphomas (12). There are two highly preferred sites (hotspots) for the *bcl-2*/J_H translocation (13,14) in

the 3' region of the *bcl-2* proto-oncogene and hotspots in the six J_H segments of the immunoglobulin heavy chain locus (15). Because of these hotspots, it is possible to establish PCR assays that can detect a large percentage of these translocations. In this study we have substituted semi-nested PCR for PCR followed by blot hybridization to amplify and visualize this translocation and we have optimized such amplification reactions and used them for quantitation. Because of the strong clustering of naturally occurring translocation breakpoints on both of the involved chromosomes, the PCR assay that we have developed can detect a large percentage of these translocations. We have used this assay to quantitate these translocations in the blood of normal individuals as well as in genotoxin-treated cultured human cells. For the human cells that were treated with known carcinogens, we also compared the frequency and spectrum of chromosomal aberrations detected by cytogenetic techniques

Due to the positions of the most frequent breakpoints for formation of the *bcr/abl* translocations associated with chronic lymphocytic leukemia (CLL), it is also possible to assay by PCR for this chromosomal rearrangement (16, 17). This translocation is implicated as one of the necessary steps of oncogenic transformation in >90% of the cases of CLL (18, 19). For PCR assay of this translocation, amplification has to be done on the product of reverse transcription (RT-PCR) because the most common breakpoints with respect to the two target genes are distant from one another at the genomic level but highly clustered at the cDNA level. As described below, we have set up this RT-PCR assay for *bcr/abl* translocations to use it on some of the same blood samples analyzed by PCR for *bcl-2/J_H* translocations.

We have applied the PCR assay for *bcl-2/J_H* translocations to in vivo-derived nucleated blood cells as well as to cultured human cells to assess the importance of these translocations and to try to detect carcinogen-linked chromosomal alterations. Our study has revealed that it is very difficult to induce the *bcl-2/J_H* translocation in cultured human cells even with a variety of carcinogens. Surprisingly, with this ultra-sensitive assay, this translocation was detected at low, but appreciable, levels in the blood of almost half of healthy adults. Therefore, conditions are favorable for the accumulation of this very early,

premalignant chromosomal alteration in vivo. Furthermore, some healthy individuals had up to a thousand-fold higher level of these translocations than others. This suggests that this assay might be used to identify people at risk for lymphoma development, especially follicular lymphoma (FL) and diffuse large cell lymphoma (DLCL). Some of these people may have unusually high levels of these translocations because of exposure to genotoxins in the course of work, such as armed forces-related or industrial work. For example, Lipkowitz and coworkers (20) found that agricultural workers exposed to pesticides used for grain storage had a highly elevated level of abnormal T-cell receptor gene chromosomal rearrangements in their T lymphocytes during periods of high exposure but not during periods of low exposure.

6. BODY OF REPORT

A. Assaying for Gene Amplification in Response to Genotoxins: Methotrexate Resistance and the Use of DNA-Repair Deficient and Normal Fibroblast Cell Lines for In Vitro Testing

(1) Cell Lines

We had planned to use for genotoxicity testing the following SV40-transformed human cell lines from Coriell Institute: xeroderma pigmentosum fibroblasts (XP; GM04312A; complementation group A; less than 2% of normal induced unscheduled DNA synthesis; karyotype, not available); "normal" fibroblasts (NF; GM00637F; from apparently normal non-fetal tissue; note that they are are SV40-transformed like others; 43% of cells diploid and 57% tetraploid); and Bloom syndrome fibroblasts (BS; GM08505A; karyotype is abnormal with multiple breaks and rearrangements; near tetraploid; reduced DNA ligase I activity). We demonstrated by cytogenetic assays that they had highly unstable karyotypes. Furthermore, these lines grew very slowly despite our attempts to optimize their medium and conditions of growth. For example, we varied the pH of the medium and found that pH 6.9 - 7.3 was optimal for culturing these two cell lines. We showed that the plating efficiency of the SV40-transformed human fibroblast cell lines was also problematic. The plating efficiency of 3T6 cells (mouse embryo-derived cell line) in medium with fetal bovine serum (FBS) vs. with dialyzed newborn calf serum (dialyzed NCS) was

43 ± 6 (n = 5) and 15 ± 2 (n =2), respectively. In another experiment, it was 31% in medium with FBS. Plating efficiencies in medium containing FBS were as follows: for BS cells, 9, 3, and 3% in three experiments; for XP cells, 7%; and for analogous NF cells, 7 and 2% in two experiments. The use of gelatin-coated plates did not increase these low plating efficiencies. For SV40-transformed human fibroblasts (NF), the plating efficiency was several fold lower when medium containing dialyzed NCS was used rather than that containing FBS. HeLa cells, however, showed the same plating efficiency in dialyzed NCS vs. in FBS. Because of these problems we abandoned the use of these repair-deficient cell lines and instead tried the much easier to handle murine cell line, 3T6 for methotrexate-resistance experiments.

(2) Methotrexate-Resistance Experiments

With 2 x 10^5 3T6 cells per 100-mm plate, we found that 324 nM methotrexate rather than 167 nM should be used to select for methotrexate resistance. Treating the 3T6 cells with phorbol ester (TPA) did not increase colony formation. At most one colony was obtained per plate subjected to the 324 nM methotrexate selection medium. This was contrary to prediction and so indicated that we were having problems getting detectable amplification of the dihydrofolate-resistance gene, which when present in multiple copies confers methotrexate resistance.

We found that the LD_{50} for methotrexate on XP cells and NF (SV40-transformed normal fibroblasts) was ~60 nM in medium with dialyzed NBS. No colonies grew up when 2 x 10^5 cells of each type were treated with 167, 250, or 324 nM methotrexate. Again, we were unable to obtain the expected background level of methotrexate-resistant colonies. We abandoned this part of the research because it took a very long time and much effort to test for the growth of these methotrexate-resistant colonies and we were not obtaining the expected starting results. Also, the PCR portion of our planned experiments, was giving us very interesting results so we focused our attention on them.

B. Development and Application of a Quantitative PCR Assay for *bcl-2/J_H* Rearrangements

(1) PCR Reagents

We developed a PCR assay for single-molecule detection of *bcl-2/J_H* translocations that was quantitative in the range of 1-50 molecules of the target DNA in the starting mixture (21). PCR was carried out in a thermal cycler (Ericomp) with primers synthesized on an Applied Biosystems synthesizer and purified by electrophoresis on a 20% polyacrylamide gel before use. For the first round of PCR to detect *bcl-2/J_H* translocations, major breakpoint region (mbr; a translocation hotspot of ~150 base-pairs [bps] within the 3' untranslated portion of the gene) and primer J_H, 5'-ACCTGAGGAGACGGTGACC-3', a consensus human J_H DNA sequence from the antisense strand of the J_H region of t(14;18) chromosomes. For the second round of PCR, we used primer J_H and radioloabeled primer 3, 5'-ACATTGATGGAATAACTCTGTGG-3', from the sense strand upstream of the *bcl-2* gene's mbr; this sequence is located 67 bps downstream of primer 1. Primer 3 (5 pmol) was labeled with [γ-^{32}P]ATP (80 μCi; 3000 Ci/mmol) in a reaction catalyzed by T4 polynucleotide kinase (20 units) and then mixed with ~300-fold excess of unlabeled primer 3 to a final specific activity of 1000-3000 cpm/pmol. The reactions were catalyzed by Taq polymerase (Promega) or the Stoffel fragment of AmpliTaq DNA polymerase (Perkin-Elmer). The intact Taq polymerase, from batch to batch, gave consistent single-molecule detection in the presence of a vast excess of nonspecific DNA unlike the latter enzyme despite the intact polymerase having some 5'-to-3' exonuclease activity. The targeted template for PCR was a t(14;18) *bcl-2*/J_H translocation at the mbr locus in DNA from a human B-lymphoma cell line, SU-DHL-4 (Oncogene Science). The concentration of the commercial SU-DHL-4 DNA stock solution was confirmed by agarose gel electrophoresis of multiple samples against sized-matched DNA standards upon visualization by fluorescence induced by ethidium bromide (EB). The background DNA that was added to the PCR mixtures to mimic a human DNA sample containing only a few copies of the translocation product in a high background of normal human DNA was normal cerebellum DNA. Contamination of the PCR mixtures was prevented by handling PCR products and reaction ingredients in separate rooms with

dedicated pipeting devices and reagents.

(2) Reaction Conditions for the First Round (20-45 Cycles) of PCR for Detection of *bcl-2/J_H* Translocations

To minimize mis-primed DNA amplification, we used the Hot Start PCR method (21) employing a paraffin wax (AmpliWax PCR Gem, Perkin-Elmer Cetus) in the first round of PCR. The 100 µl-reaction mixtures consisted of different numbers of copies of SU-DHL-4 DNA containing the target translocation; 2.5 units of Taq polymerase (Promega) or 10 units of the Stoffel Fragment of AmpliTaq; dATP, dGTP, dCTP, dTTP (200 µM each); 2.5 mM $MgCl_2$; 20 pmol each of primers 1 and J_H; 7 µg of normal human brain DNA; and 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100, 50 mM KCl (for the Promega enzyme) or 12.5 mM Tris-HCl, pH 8.3, 12.5 mM KCl (for the Stoffel fragment). The initial denaturation step was at 95°C for 5 min followed by the indicated number of cycles of 1 min of annealing at 60°C, 1 min of primer extension at 72°C, and 1 min of denaturation at 94°C. In the last cycle, the primer extension time was 10 min. The PCR products were analyzed by electrophoresis of 30 µl of the reaction mixture on a 5% polyacrylamide gel and were visualized by their ethidium bromide-induced fluorescence.

(3) Reaction Conditions for the Second Round (20 Cycles) of PCR

For the second round of PCR, 5 µl of the undiluted product obtained from the first round of PCR was used in a reaction mixture that was the same as that for the first round except that radiolabeled primer 3 (~2000 cpm/pmol) replaced unlabeled primer 1 and the total reaction volume was 75 µl. Given the much lower sequence complexity of this reaction mixture, to economize, Ampliwax was not used in the second round of PCR. Rather, the mixture was overlaid with two drops of mineral oil and kept on ice until after the 5-µl sample from the first-round PCR product was added through the oil layer to the aqueous layer. The DNA was then subjected to an initial denaturation for 5 min at 95°C followed by 20 cycles of 1 min at 60°C, 1 min at 72°C, and 1 min at 94°C except that the final primer extension was for 10 min at 72°C. The products from the second round of PCR were analyzed by electrophoresis, as above, followed by

autoradiography. For quantitative PCR, the specific DNA fragments (~330- and, when observed, 830-bp products) were excised from the gel and their Cerenkov radiation (i.e., light emission in the absence of added scintillants) was measured.

(4) Quantitation of *bcl-2/J_H* Translocations with this PCR Assay

We optimized conditions for PCR detection of a few copies of a DNA containing a chromosome 14/18 translocation at the *bcl-2* proto-oncogene in a background of DNA from approximately 10^6 normal human cells (7 μg). The source of the translocation-containing DNA was SU-DHL-4 cells in which this t(14;18) rearrangement occurs between the translocation-hotspot mbr locus in the 3'-untranslated region of the *bcl-2* proto-oncogene and the fourth of six related J_H regions at the heavy chain immunoglobulin locus. Normal human DNA should give no detectable specific amplification products because the primer-complementary sites in the genomic DNA are on separate chromosomes. After determining that 2.5-5.0 mM Mg^{+2} ions and 60-64°C were optimal for the maximum sensitivity and specificity, we tried to detect only a few copies of the specific translocation product after 40 cycles of PCR with a single pair of primers (primers 1 from chromosome 18 and J_H from chromosome 14). Such primers can amplify a high percentage of naturally occurring, lymphoma-associated translocations (11, 14). We could detect, by EB-induced fluorescence, a specific amplification product of the expected size, ~400 bps, from samples with only two copies of a t(14;18) chromosome in a background of 7 μg of normal human DNA (data not shown). After 30 cycles of PCR with the same J_H primer and a different mbr-specific primer using Southern blotting to visualize the specific products, Negrin *et al.* (22) obtained a similar specific DNA fragment from as little as one copy of SU-DHL-4 DNA in a background of DNA from 10^5 normal cells. However, when we visualized the PCR products by ethidium bromide-induced fluorescence, the large amount of background normal human DNA in the samples gave rise to about six bands of nonspecific amplification products.

It was desirable to eliminate the background of nonspecific amplification bands so that the procedure could be used to detect newly arising t(14;18) amplification products whose exact size could

not be predicted. The most reproducible, specific, and sensitive method for the detection of only a few molecules of the target DNA in a background of 7 μg of nontarget DNA was semi-nested PCR for two rounds using 22 cycles of amplification with unlabeled primers 1 and J_H in the first round and then 20 cycles with unlabeled primer J_H and labeled primer 3 in the second round (semi-nested PCR). The main specific radiolabeled product obtained from these semi-nested reactions containing as little as one target DNA molecule was a ~330-bp fragment clearly visible after only a single day of autoradiographic exposure. Sometimes a secondary product of ~830 bps, apparently from priming at the J5 rather than the nearer J4 sequence on SU-DHL-4 DNA, was seen (data not shown) as reported by Negrin et al. (18). No radiolabeled products were ever observed in the samples that contained only the background normal human DNA.

Using the above optimal conditions for semi-nested PCR, we determined the relative amounts of radioactivity in the specific radiolabeled PCR products when an average of 1 to 15 target DNA molecules were present in the samples in a total of 3 to 12 independent PCR amplifications. Such replicate determinations were especially important in view of stochastic fluctuation at these extremely low copy numbers. The average amount of specific PCR product was nearly linear in the range of ~1 to 15 target molecules in the starting reaction (21). The proportionality of the average initial copy number of target molecules to the amount of specific product was further examined by a limiting dilution analysis. We compared the intensity of the ~330-bp band after the second round of PCR when the products from the first round of PCR were subjected to serial dilutions before the second round of PCR amplifications. The results were as expected and our data followed the expected Poisson distribution (21).

(5) Detection of Very Low Levels of *bcl-2*/J_H Translocations in Human Blood. We used the above highly sensitive PCR assay on DNA from peripheral blood mononuclear cells (PBM) to detect lymphoma-associated translocations between the MBR, the breakage hotspot of the *bcl-2* gene on chromosome 18, and the J_H region on chromosome 14 in the blood samples from non-cancerous individuals (23). B

lymphocytes in the PBM fraction are expected to be the source of *bcl-2*/J_H translocations because the DNA sequences (including N-type insertions) at the junction points of these translocations from PBM, follicular lymphomas, pre-B cell leukemias, and hyperplastic tonsils indicate that these translocations occur during B cell differentiation (24-26 and see below).

In the present study, most of the 132 blood samples, including all samples from individuals between the ages of 18 and 50, came from healthy blood-bank donors and the rest were from patients with diseases other than lymphoma. In semi-nested PCR (Fig. 1A) on DNA from these blood samples, any t(14;18) chromosomes whose translocations occurred between the MBR of the *bcl-2* gene and one of the six J_H regions should give amplification products of about 0.6 to 0.8 kilobase-pairs (kb). Even a single translocation-containing DNA molecule can be visualized in this assay (21). Of the tested PBM samples, which contained a total of 7-35 μg of DNA representing approximately 1-5 x 10^6 cells, 47% gave specific PCR products in the expected size range (bcl/J^+ DNA samples; Fig. 2). The standard used in all PCR experiments was DNA from SU-DHL-4, a diffuse histiocytic lymphoma-derived cell line whose t(14;18) chromosome apparently contains a deletion of several hundred base-pairs in the MBR/J_H4 junction region (21). Upon semi-nested PCR, the translocation target sequence gives rise to an ~0.3-kb band instead of 0.6 to 0.8 kb (Fig. 2A, lanes 9-11) and, sometimes to a secondary higher-molecular-weight product (Fig. 2A, lane 11). This secondary band is probably due to the consensus sequence J_H primer sometimes priming at J_H5 instead of J_H4.

In all sets of PCR assays, control reactions were performed with a human brain DNA sample that contains no detectable *bcl-2*/J_H translocations. These mixtures never gave a band in the 0.2- to 1.0-kb region (Fig. 2A, lane 8). Similarly, the PBM samples that showed no bands in the expected region for translocation products usually had no labeled bands at all (including no non-specific labeled bands) after amplification (Fig. 2A, lanes 1-3, 6; Fig. 2B, lanes 7-10).

(6) Analysis of the Translocation Frequency with Respect to Age, Gender, and Health Status of the

Donors. Follicular lymphoma, the cancer most frequently associated with the *bcl-2/*J_H translocation, usually occurs in people over the age of 60 and only rarely in those less than 40 (27). In this study, there was a statistically significant linear trend in the proportion of translocation-positive blood donors with age. The percentages of translocation-positive individuals in the 6-20, 21-40, 41-60, and ≥61 age groups (including the non-lymphoma cancer patients) were 14%, 42%, 62%, and 60%, respectively ($X_1^2 = 11.7$; $P < 0.01$). Moreover, we observed a significant steady increase with age ($X_1^2 = 14.2$; $P < 0.001$) in the percentage of individuals having rather high translocation frequencies, namely, ≥20 translocations per 5 x 10^6 PBM, and this relationship was linear. This linear trend was also statistically significant when the non-lymphoma cancer patients or sickle cell patients were excluded.

The correlation between *bcl-2/*J_H translocation frequency and the age of each blood donor (Fig. 3) was analyzed by the Spearman's rank correlation test. We observed a statistically significant increase with age ($r = 0.37$; $P < 0.001$; excluding the sickle cell patients, $r = 0.25$; $P < 0.001$). The Spearman rank correlation is not strongly affected by extreme values, such as that from the 35 M PBM sample (Fig. 3). In contrast, when analyzing the data by Pearson's correlation test, a statistically significant linear correlation was not seen unless this outlier was excluded from the analysis. In addition to this 35-yr-old male, two other healthy blood donors less than 40 years of age had rather high levels of translocation-bearing PBM (25 M and 29 F, Fig. 3). Therefore, much higher-than-normal levels of *bcl-2/*J_H translocations in PBM of a small portion of apparently cancer-free individuals is seen in younger adults as well as in older adults despite the general relationship between translocation frequency and age.

In addition, a significant correlation was observed between those donors who had multiple-sized *bcl-2/*J_H translocation products and age (Spearman rank $r = 0.32$, $P < 0.001$). That 3 out of 43 people between the ages of 21 and 40 appear to have three or four independently generated bcl/J^+ PBM populations while the majority (58%) of this age group had no detectable translocations (23) suggests that some individuals may be especially prone to generating or accumulating multiple independently arising

bcl-2/J$_H$ translocations. Furthermore, individuals who are more likely than normal to rearrange their *bcl-2* and immunoglobulin gene loci might also be more likely to generate cells with illegitimate recombination products involving other proto-oncogenes and so be also at risk for certain types of cancer in addition to those associated with *bcl-2/J*$_H$ translocations.

With regard to the health status of the blood donors, all the PBM samples from individuals between the ages of 18-50 were from volunteer blood donors, prescreened for good health. Most of the elderly blood donors were clinic patients with diseases other than cancer. Those cancer patients included in Fig. 3 did not have types of cancer associated with *bcl-2/J*$_H$ translocations. The translocation data obtained from PBM of the small number of over-60 non-lymphoma cancer patients (seven) were similar to the data obtained from the other over-60 donors (23). We did analyze one sample from a patient with recurrent lymphoma, who was not currently showing signs of the disease. This individual had only 7 *bcl-2/J*$_H$ translocations per 5×10^6 PBM and no evidence for multiple independent translocations.

A higher percentage of translocation-positive adults were found among males than among females. The percentages of individuals who were bcl/J$^+$ in the 21-40, 41-60, or over-60 age groups for males (M) *vs.* females (F) were as follows: M, 55% *vs.* F, 29%; M, 71% *vs.* F, 56%; and M, 73% *vs.* F, 54%, respectively. Furthermore, a similar gender difference was observed for subjects with high translocations frequencies. The percentages of individuals with ≥20 translocations per 5×10^6 PBM in the 21-40, 41-60, or over-60 age groups for males *vs.* females were as follows: M, 18% *vs.* F, 5%; M, 14% *vs.* F, 11%; and M, 46% *vs.* F, 33%, respectively. Although the numbers of individuals studied here were insufficient for achieving statistical significance, these results suggest that males are more prone to accumulating these translocations than females, which might be related to males having a higher rate of non-Hodgkin's lymphoma than females (34).

(7) In Vitro Testing by PCR for Genotoxin-Induced *bcl-2/J*$_H$ Translocations and by G-Banding of Metaphase Chromosomes for General Chromosomal Rearrangements

Using standard techniques for G-banding metaphase chromosomes (28), we compared the frequency of various types of gross chromosomal rearrangements in 50 metaphases from cultured human cells treated with a variety of genotoxins to the PCR-determined level of induced *bcl-2/J_H* translocations. For this comparison we used a diploid human cell line, FLEB14 cells, a pro-B cell line. Table 1, which summarizes data from many experiments, shows that only in one experiment did we detect bcl-2/J_H translocation-containing molecule. This was not an artifact of chemical contamination because in hundreds of PCR assays with negative controls (only translocation-free human brain DNA) we never obtained a false positive. Despite this extremely low frequency of *bcl-2/J_H*-positive cells (<1 per 4 x 10^7 treated cells), 10-18% of these treated cells showed the presence of a variety of chromosomal rearrangements detectable by G-banding of metaphases (Table 1). This pro-B cell line has not yet rearranged its immunoglobulin genes and so might have been a suitable cell type for genotoxin-induced immunoglobulin gene rearrangement mistakes, like generation of the *bcl-2/J_H* translocation. However, this was not the case.

We also tried to induce *bcl-2/J_H* translocations in two cell lines with DNA repair deficiencies, namely, BS and XP fibroblast cell lines described above. There was no detectable *bcl-2/J_H* rearrangement in these cell lines as determined by our PCR assay with single-molecule sensitivity (Table 2). In these cell lines it was too difficult to assess the level of overall chromosomal rearrangements, as we had done for FLEB14 cells because of their unstable and highly aneuploid karyotypes.

(8) Frequency of *bcl-2/J_H* Translocations in Peripheral Blood of Follicular Lymphoma Patients

About 85% of follicular lymphomas (FLs) contain the *bcl-2/J_H* translocation [t(14;18)(q32;q21)] between the *bcl-2* proto-oncogene and the J_H immunoglobulin gene regions (25). Although recent PCR methods for detecting these translocations in patients' bone marrow and peripheral blood (21) might allow monitoring of the effectiveness of treatment and the progress of the disease, their prognostic significance is controversial (11,15). Also, as a complicating factor, healthy individuals often contain PCR-detectable translocations in their circulation at levels ranging from 1- 1000 translocations per 5 x

10^6 peripheral blood mononuclear cells (PBM; 23).

Little data are available on the quantitation by PCR of these translocations in peripheral blood of follicular lymphoma (FL) patients. In order to better understand the biological significance of the *bcl-2/J_H* translocations that we detected in the blood of almost half of the normal adults that we studied, we have begun assessing the level of these translocations in peripheral blood samples from FL patients because these patients usually have this translocation in their tumors. By the above-described quantitative PCR, we assayed for these translocations in PBM DNA from five FL patients (Zhang et al., in prepn.). Only one had a high level of these translocations in the circulation, namely, about 35,000 translocations per 5 x 10^6 PBM. Although he displayed an excellent performance ratio at the time of this assay, this patient died one month later. This indicates that abnormally high levels of these translocations in the circulation are sometimes associated with FL.

C. Assaying for *bcr/abl* Translocations by RT-PCR

We have set up a sensitive reverse transcription PCR (RT-PCR) assay for chromosomal rearrangements involving proto-oncogenes *bcr* and *abl* in Ph^1 positive cells (3). This method will be applied to detect *bcr-abl* translocation levels (percentage of Ph^1 positive cells) in blood or bone marrow of chronic myelogenous leukemia (CML) patients and in cloned mononuclear cells of CML patients after different combinations of chemotherapy and immunological purging. About 95% of CML patients have this translocation. The translocation activates the c-*abl* proto-oncogene thereby contributing to oncogenesis. This translocation can be assayed by RT-PCR, but not by PCR of genomic DNA because of the scatter of the translocations breakpoints, which, at the cDNA level are no longer disparately positioned (3). We will use this RT-PCR assay to determine whether cancer-free individuals with high frequencies of *bcl-2/J_H* translocations in their peripheral blood also have significantly higher than average frequencies of the *bcr/abl* translocation. In the blood of healthy adults, *bcr/abl* translocations are detectable although normally at very low levels (29).

We optimized RT-PCR conditions based upon the method of (16) using total RNA isolated from a Ph^1 positive cell line (K562) and Ph^1-negative human B lymphoblast cell line (G1) We were able to detect the specific fused *bcr-abl* mRNA derived from trancription of the translocation at high sensitivity. Using a diluted sample containing RNA from an average of only 0.5 translocation-positive K562 cells in a background of RNA from 5 x 10^4 translocation-negative G1 cells we could detect the expected sized PCR product (~375 bp) by ethidium bromide-induced fluorescence. This was the only detected PCR product of the amplification.

7. CONCLUSIONS

Semi-nested PCR allowed routine detection of a single translocation-containing molecule of human DNA in the presence of DNA from approximately 5 x 10^6 normal human cells and quantitation of the levels of these translocations. Because of the sensitivity and specificity of this methodology, it can be applied to various types of cultured human cells treated with mixtures of military waste products whose carcinogenic potential is being evaluated and the ability of such samples to induce this type of cancer-causing translocation can be determined and compared to their cell toxicity.

Follicular lymphomas or diffuse large-cell lymphomas that progress to high grade lymphomas are often associated with *bcl-2/J_H* translocations throughout the tumor (1). These cancers contribute towards the ~19,000 deaths per year from non-Hodgkin's lymphoma in the U.S. That approximately 43% of healthy blood donors had detectable *bcl-2/J_H* translocations in their peripheral blood cells indicates that the mere presence of such translocations is not a risk factor for follicular lymphoma. However, the level of these translocations in PBM from apparently healthy people varied over almost three orders of magnitude with a general age-dependence that may be one of the sources of the strong age-dependence of the occurrence of follicular lymphomas.

Furthermore, our finding that 3 out of 57 healthy blood donors of less than 40 yr of age had more than 50 translocations per 5 x 10^6 PBM suggests the existence of a subpopulation of adults at risk

for follicular lymphoma or diffuse large-cell lymphoma later in life. Because these cancers are rare before middle age, the possibility that these individuals already have occult lymphoma and, hence, a high translocation frequency in their PBM can be discounted. Instead, we hypothesize that environmental factors predispose some adults before middle age to accumulating *bcl-2/J_H* translocations in PBM due to aberrant immunoglobulin gene maturation and this could lead to cancer if certain other genetic changes occur in these cells. Evidence for heavy smoking being associated with an increase in the *bcl-2/J_H* translocation frequency was recently reported (30). Although we have not yet been able to induce formation of these lymphoma-associated translocations in cultured human cells, our PCR assay of peripheral blood samples has provided evidence we may be able to detect people at risk for lymphoma, and possibly other types of translocation-associated cancers, because of exposures to work-related genotoxins.

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9. APPENDIX

See following pages of figures and tables referred to in text.

10. BIBLIOGRAPHY, MEETING ABSTRACTS AND LIST OF PERSONNEL

Published Papers

X.-Y. Zhang, & M. Ehrlich. Detection and quantitation of low numbers of chromosomes containing *bcl-2* oncogene translocations using semi-nested PCR, BioTechniques **16**: 502-507 (1994).

W. Ji, G.A. Qu, P. Ye, X.-Y Zhang, S. Halabi, & M. Ehrlich. Frequent detection of *bcl-2/J_H* translocations in human blood samples by a quantitative PCR assay, Cancer Research **55**: 2876-2882 (1995).

Meeting Abstracts

Gordon Research Conference on "DNA Alterations in Transformed Cells," invited talk on "Frequent detection of *bcl-2/J_H* translocations in blood from normal individuals and cancer patients," 6/30/96 -7/4/96, Tilton, New Hampshire

Personnel

Dr. Xian-Yang Zhang

Weizhen Ji

Alan Qu

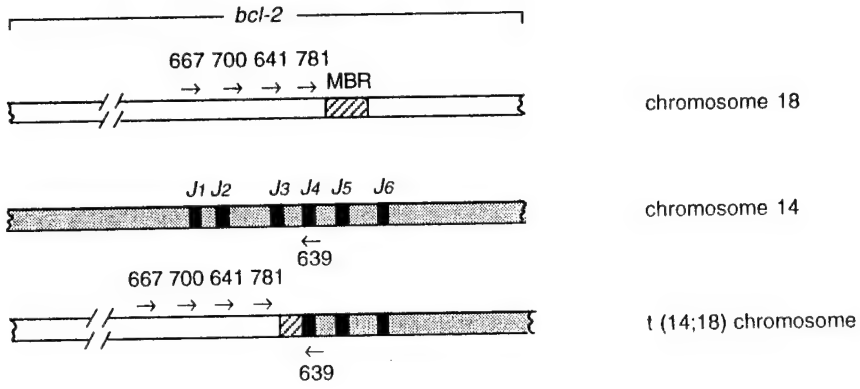
Peng Ye

Anne Johnson

Richi Tawa

bcl-2/J$_H$ TRANSLOCATIONS IN BLOOD OF HEALTHY DONORS

A



B

PBM Sample	*bcl-2* MBR	Intervening region	J_H	
30 M	CTGCCCTCCTTCCGC	ACCGATATATGGA	TACTACTACGGTATGGACGTCTGGGGCCAAGGGACACGGTCACCGTCTCCTC	*J6*
70 M	CCTGAAATGCAGTGGTGCT	CTCACCCTTAAACCTCGTTAGGCTTATCTCCTTTCAACCTCTC	CTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTC	*J4*
55 F a	GTGGTATGAAGCC	GATAGCATAGTCCCTAACTGCGAAACACGACACTGCGACGACTGCACGTCCGCTCAGACGGTTTA	TTGATGTCTGGGGCCAAGGG	*J3*
55 F b	TGCAGTGGTGCTTA	ATGACCAACTGGTTCGACC	CTGGGGCCAGGGCAACCCTGGTCACCGTCTCCTC	*J1*

FIG. 1 Detection of *bcl-2/J*$_H$ translocations by semi-nested PCR and analysis of the translocation junctions in two translocation-positive DNA samples from the blood of healthy individuals. A, the relative position of sequences complementary to the primers (not drawn to scale) used for semi-nested PCR to detect translocations between J_H loci and the major breakpoint region (MBR) at the end of the *bcl-2* gene. Usually, in the first and second rounds of PCR, primer pairs #667/639 were employed, respectively, with ^{32}P-labeled primer 641 as the source of radioactivity. To check that the PCR products were derived from the *bcl-2/J*$_H$ translocation, primer #641 was replaced with primers #700 or 781. In semi-nested PCR, these sets of primers yield labeled products only from the *bcl-2/J*$_H$ translocation-bearing chromosome. Primer #639 is shown annealing to part of the J_H sequence but this J_H consensus sequence primer can appeal to any of the six J_H sequences. B, the DNA sequences at the junction region of PBM samples from three healthy blood donors. In the case of the 55 F amplification products, 55 F a and b refer respectively to the 880- and 720-bp PCR products which were separately sequenced.

From Ji et al., Cancer Res. 55: 2876-2882, 1995.

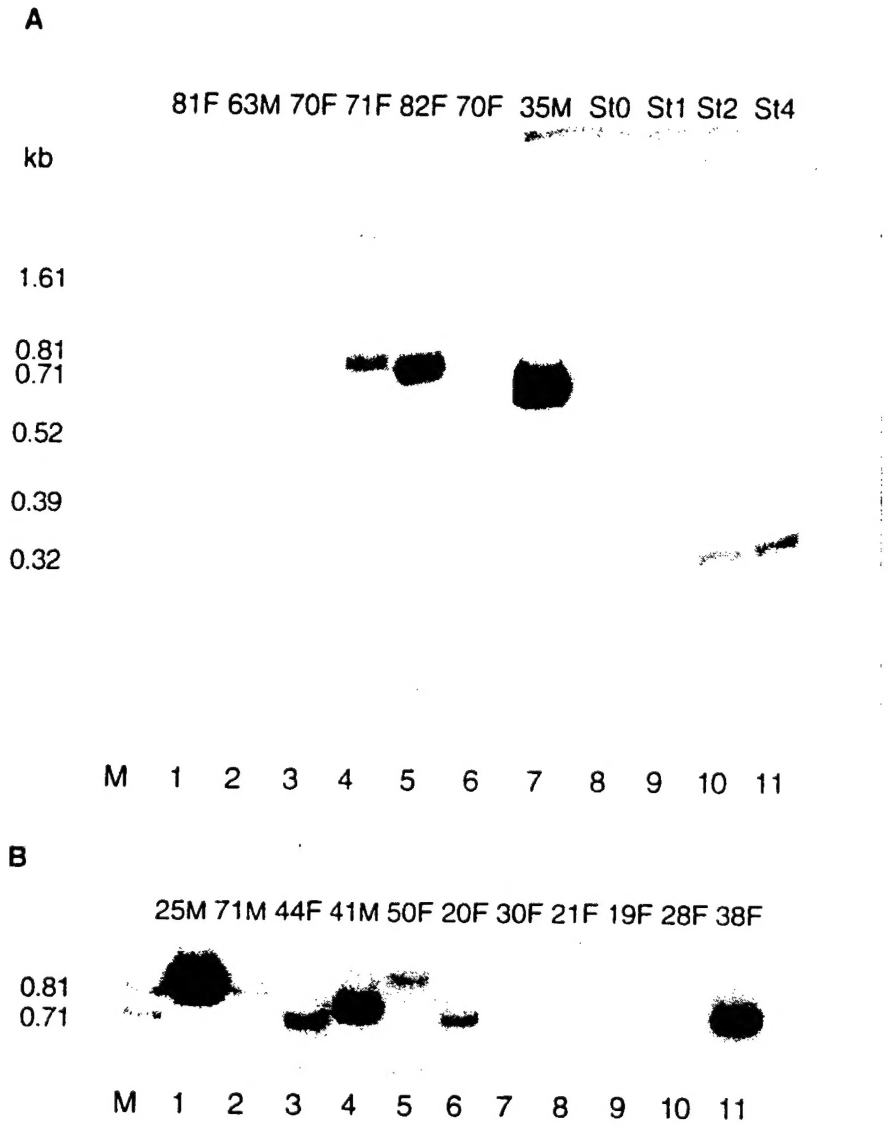


FIG. 2 Characterization of *bcl-2/J_H* translocation-derived PCR products from Blood samples. Fourteen of 7 μg of PBM DNA from 18 individuals were amplified, and the PCR products were electrophoresed on a 6% polyacrylamide gel and visualized by autoradiography. The age and sex of each blood donor is indicated. A, Lanes 4, 5, and 7, and B, Lanes 1-6, and 11, translocation-positive (*bcl-2/J_H^+*) PBM samples. A, Lanes 1-3 and 6, and B, Lanes 7-10, translocation negative PBM samples. A, Lanes 9-11 (Stl, St2, and St4), aliquots of a *bcl-2/J_H^+* standard, SU-DHL-4 DNA, that should contain an average of 1, 2, or 4 molecules of the bcl-2/J_H translocation; Lane 8 (St0), 14 μg of human brain DNA alone, as a negative control, subjected to PBM samples. The amplification experiment in A and that in B were done with different batches of ^{32}P-labeled primer; therefore, they are not comparable. M, molecular weight DNA markers.

From Ji et al., Cancer Res. 55: 2876-2882, 1995

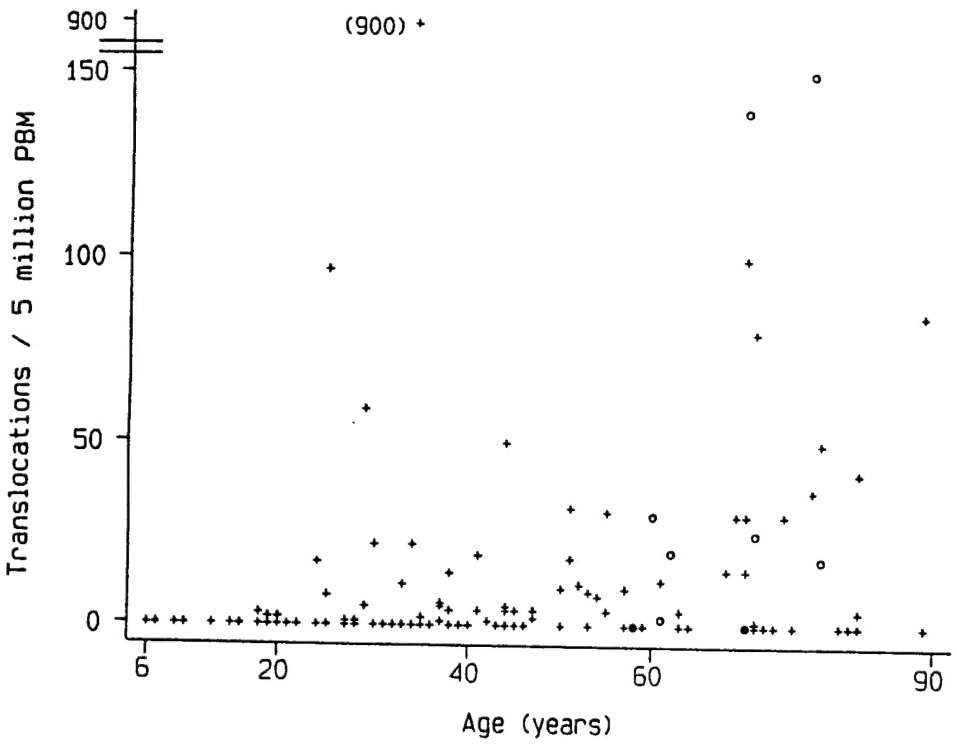


FIG. 3 Number of *bcl-2/J$_H$* translocations per 5 x 10^6 mononuclear cells from peripheral blood vs. the age of the donor. The number of MBR-typed *bcl-2/J$_H$* translocations per 5 x 10^6 PBM (35 μg of DNA) is plotted vs. the age of the blood donor. The off-scale translocation frequency of the 35-yr old male, donor #69, who had 900 translocations per 5 x 10^6 PBM, is indicated +, healthy donor or non-cancer patient; °, cancer patient.

From Ji et al, Cancer Res. 55: 2876-2882, 1995

Table 1.

Induction of chromosomal aberrations in a pro-B cell line (FLEB14) treated with various genotoxins

Treatment	Frequency of chromosomal aberrations per metaphase		*bcl-2/J_H* translocation analysis	
	Chromosomal breakage products	Chromosomal rearrangements	Amount of DNA examined (µg)	Frequency per 2 x 10^6 cells
0.1 µg/ml $K_2Cr_2O_7$	0.04	0.10	14	0
0.2 µg/ml $K_2Cr_2O_7$	0.04	0.18	14	0
0.3 µg/ml $K_2Cr_2O_7$	0.05	0.12	28	0
0.5 µg/ml $K_2Cr_2O_7$	0.04	0.14	14	1
0.7 µg/ml $K_2Cr_2O_7$	0.14	0.18	14	0
0.05 µg/ml MNNG	0.02	0.10	35	0
0.2 µg/ml MNNG	0.06	0.10	35	0
0.5 µg/ml MNNG	0.06	0.08	35	0
0.5 µM azaCR	0.24	0.18	35	0
0.5 µM azaCR + 0.05 µg/ml MNNG	0.20	0.14	35	0
0.5 µM azaCR + 0.2 µg/ml MNNG	0.24	0.14	35	0
no genotoxin	<0.02	<0.02	112	0

[1] The diploid human cell line, FLEB14, was treated for 1 h with potassium dichromate or MNNG and 18 h with 5-azacytidine followed by incubation in normal medium for 3 d prior to harvesting cells for assessment of chromosomal rearrangements by G-banding of metaphase chromosomes or of *bcl-2/J_H* translocations by PCR assay of DNA (21).

Table 2.

Assays for *bcl-2/J_H* translocations in cultured human cells treated with MNNG or azaCdR

Cell line	Control (DMSO)	No. of *bcl-2/J_H* translocations detected / No. of cells treated				
		0.1 µg/ml MNNG	0.5 µg/ml MNNG	1.5 µM azaCR	1.5 µM azaCR plus	
					0.1 µg/ml MNNG	0.5 µg/ml MNNG
BS	0/1.8 x 10^7	0/2.5 x 10^7	ND	0/1.6 x 10^7	0/2.5 x 10^7	ND
XP	0/2 x 10^7	0/2 x 10^7	ND	0/2 x 10^7	0/2 x 10^7	ND
NF	0/2.4 x 10^7	0/1.2 x 10^7	0/1.2 x 10^7	0/2.4 x 10^7	0/1.2 x 10^7	0/1.2 x 10^7

[1] Exponentially growing cells were treated with MNNG for 1 h; azaCR for 18 h; or azaCR for 1 h, incubated in normal medium for 5 h, and then treated with MNNG for 1 h. Cells were harvested 3 d after treatment to isolate DNA and test for MBR-type *bcl-2/J_H* translocations by PCR.